

Synthetic Phenolic Antioxidants and Their Metabolites in Follicular Fluid and Association with Diminished Ovarian Reserve: A Case–Control Study

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BACKGROUND: Diminished/decreased ovarian reserve (DOR) is a disorder of ovarian function, which severely affects women's reproductive health. Accumulating evidence has found that adverse environmental factors can affect ovarian function. However, whether synthetic phenolic antioxidants (SPAs) exposure is associated with DOR is still unknown.

OBJECTIVES: We explored whether concentrations of SPAs and their metabolites are associated with DOR.

METHODS: A case–control study was conducted from January 2019 to January 2020 in China. One hundred eighty-one women 20–44 years of age, with (case group, $n = 63$) and without DOR (control group, $n = 118$) were included in our study. The follicular fluid concentrations of typical SPAs and their metabolites were measured, including butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and five BHT metabolites [3,5-di-*tert*-butyl-4-hydroxy-benzylalcohol (BHT-OH), 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (BHT-CHO), 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT-COOH), 2,6-di-*tert*-butyl-1,4-benzoquinone (BHT-Q), and 2,6-di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one (BHT-quinol)]. Information about serum basal concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), and anti-Müllerian hormone (AMH) and the basal antral follicle count (AFC) was collected.

RESULTS: The measured frequencies of BHA, TBHQ, BHT, BHT-OH, BHT-CHO, BHT-COOH, BHT-Q, and BHT-quinol in follicular fluid were 1.7%, 2.2%, 40.3%, 46.4%, 57.5%, 100%, 64.6%, and 49.2%, respectively. The concentrations of BHT-CHO (0.49 ng/mL vs. 0.12 ng/mL, $p = 0.041$), BHT-COOH (0.45 ng/mL vs. 0.28 ng/mL, $p < 0.001$), BHT-Q (0.70 ng/mL vs. 0.13 ng/mL, $p < 0.001$), and the sum of five BHT metabolites (Σ metabolites; 1.79 ng/mL vs. 1.0 ng/mL, $p < 0.001$) in the case group were significantly higher than those in the control group. The risk of DOR was further analyzed according to the tertiles of chemical concentration. Compared with the low levels of BHT metabolites, the adjusted odds ratios (ORs) for DOR were significantly increased in the high levels of BHT-CHO [OR = 3.19, 95% confidence interval (CI): 1.22, 8.31, $p = 0.018$], BHT-COOH [OR = 4.73 (95% CI: 1.63, 13.71), $p = 0.004$], and BHT-Q [OR = 4.48 (95% CI: 1.69, 11.86), $p = 0.003$] after adjusting for age, body mass index, education, infertility type, triglycerides, and total cholesterol. Moreover, compared with the low level of Σ metabolites, increased adjusted ORs for DOR were found both in the middle level [OR = 4.11 (95% CI: 1.44, 11.75), $p = 0.008$] and high level [OR = 5.51 (95% CI: 1.81, 16.77), $p = 0.003$], showing an obvious dose–response relationship ($p_{\text{Trend}} = 0.003$).

CONCLUSION: In this study, we report the measured frequency and concentrations of BHA, TBHQ, BHT, and their metabolites in follicular fluid. Moreover, we found the concentrations of BHT metabolites, especially BHT-CHO, BHT-COOH, and BHT-Q, are positively associated with the increased risk of DOR. <https://doi.org/10.1289/EHP11309>

Introduction

Ovarian reserve refers to the number and quality of follicles and oocytes, indicating a woman's reproductive potential. Diminished/decreased ovarian reserve (DOR) is a condition in which the number and quality of oocytes are reduced.¹ In clinical practice, decreased

serum anti-Müllerian hormone (AMH) level, reduced antral follicle count (AFC), and increased serum follicle-stimulating hormone (FSH) level are recognized as the indications for DOR.^{2,3} Women with DOR are more likely to suffer from reduced fecundity,⁴ early occurrence of menopause,⁵ increased miscarriage rates,⁶ and poor response to ovarian stimulation in assisted reproduction treatments,⁷ severely affecting women's reproductive health. It is reported that DOR prevalence has been increasing in recent years worldwide.⁸ However, the etiology of DOR is still unclear. Although age is an independent risk factor for DOR,^{3,9} a growing body of studies has recently revealed that environmental factors also play roles in the development of DOR.^{10–12}

Synthetic phenolic antioxidants (SPAs), including butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), and butylated hydroxytoluene (BHT) are an emerging group of anthropogenic chemicals that are widely used in foodstuffs, cosmetics, and plastics to retard oxidative reactions and lengthen product shelf life.^{13,14} SPAs and their metabolites are ubiquitous in various environmental matrices, including dust,¹⁵ sea sediment,¹⁶ and river water.^{17,18} Humans are inevitably exposed to these chemicals through contact, ingestion, and inhalation of the contaminated matrices.¹⁸ The reported estimated total daily intake of BHT in adults was 0.21–372 $\mu\text{g}/\text{kg}$ body weight (BW) per day,¹⁹ with some of intake levels exceeding the acceptable daily intake level

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(250 µg/kg BW per day) recommended by the European Food Safety Authority (EFSA).²⁰ Moreover, recent studies reported these chemicals could be measured at high detection rates in human serum,^{21,22} urine,^{19,23} placenta,²¹ breast milk,²⁴ and fingernails.²⁵ This implies SPAs are prevalent in humans. However, few studies have investigated the exposure levels of these SPAs in ovarian follicular fluid.

The potential reproductive toxicity resulting from SPAs and their metabolites is of particular concern. These chemicals are recognized as endocrine disruptors, and previous *in vitro* and *in vivo* studies have demonstrated that they could disturb the homeostasis of the endocrine system by influencing the expressions of estrogen-responsive genes, transcriptional levels of steroidogenic enzymes, and ovarian steroidogenesis.^{26–28} *In vitro* studies also indicated they could cause oxidative stress, DNA damage, and apoptosis in different cells, including mouse Leydig cells, Burkitt's lymphoma cell line BJAB, and the human myelogenous leukemia cell line HL 60.^{29–31} Moreover, in cultures of rat oocyte cumulus complexes, BHT treatment can inhibit the spontaneous resumption of meiosis and impair oocyte development.³² These adverse effects resulting from SPAs and their metabolites—including endocrine-disrupting effects, oxidative stress, DNA damage, apoptosis, and reproductive toxicity—may contribute to the pathogenesis of DOR. Therefore, we hypothesize that SPAs exposure could be involved in the development of DOR. However, to the best of our knowledge, no epidemiological study has investigated this.

Therefore, in the present case-control study, we aimed to describe the measured frequencies and concentrations of SPAs in human follicular fluid and to explore whether SPAs and their metabolites are associated with DOR. We believe this study will provide important evidence for possible adverse effects of SPAs on female reproductive health, as well as provide insights into the prevention of DOR.

Methods

Study Design and Participants

This case-control study was conducted at the Center of Reproductive Medicine, Peking University Third Hospital (Beijing, China) from January 2019 to January 2020. This study was approved by the institutional review board of Peking University Third Hospital (2019SZ-023).

Infertile patients aged 20–44 with plans to undergo *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) were eligible to participate in our study. Among them, women diagnosed with DOR and without other infertility factors were eligible as cases, and women getting treatment due to only tubal factor or male factor were eligible as controls. The diagnostic criteria for DOR in our study have been described previously.¹ Briefly, women were diagnosed to have a DOR if they met at least two of the following three criteria: *a*) basal AFC <7, *b*) basal FSH level >10 IU/L, and *c*) AMH level <1.1 ng/mL on a random day of the menstrual cycle. Patients (including cases and controls) were excluded from the study if they had other conditions that may affect ovarian reserve, such as polycystic ovary syndrome, ovarian surgery, endometriosis, and genetic factors.

Recruitment Process and Informed Consent

Infertile women who came to the outpatient clinic were screened by doctors and nurses. Women who met the inclusion criteria and did not have the characteristics of the exclusion criteria were informed of the study, including background, objectives, and implementation process. Women who were willing to participate

in this study were further contacted by a researcher of our team before the oocyte retrieval process of their IVF or ICSI treatment and informed of the detailed information about the study in person.

Moreover, besides the information of the study, all participants had been informed that *a*) they had the right to refuse to participate in and to withdraw from this study anytime without any reason, *b*) the refusal/withdrawal would not have any influence on the normal treatment of IVF or ICSI, and *c*) their personal information would be protected and would not be disclosed to others.

Women who *a*) fully understood the study, *b*) agreed to participate in this research, *c*) allowed us to collect data from their medical records, and *d*) agreed to donate their follicular fluid samples for this study were asked to sign the informed consent document. All the participants included in our study signed informed consent before follicular fluid collection.

Oocyte Retrieval and Follicular Fluid Collection

All the women in our study were given gonadotropin-releasing hormone (GnRH) antagonists or GnRH agonists for controlled ovarian hyperstimulation, which have been described previously.³³ When at least two follicles reached 18 mm in diameter, 250 µg of recombinant human chorionic gonadotropin (r-hCG; Eisner) was administered to trigger oocyte maturation. At 34–38 h after r-hCG administration, oocytes were aspirated. During oocyte retrieval, follicular fluid was collected from the largest preovulatory follicle of each patient using follicle aspiration under ultrasonographic control by experienced nurses. The ultrasound equipment (F37; Hitachi Aloka Medical) was used during oocyte retrieval. Only the first dish of follicular fluid without blood contamination was collected and used in the subsequent protocol. Then the supernatants of the centrifuged samples were collected and stored at –80°C until analysis.

Measurement of Follicular Fluid SPAs

Eight target chemicals in follicular fluid samples were measured, including BHA, TBHQ, BHT, and five metabolites of BHT [3,5-di-*tert*-butyl-4-hydroxy-benzylalcohol (BHT-OH), 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (BHT-CHO), 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT-COOH), 2,6-di-*tert*-butyl-1,4-benzoquinone (BHT-Q), and 2,6-di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one (BHT-quinol)].

Briefly, for each 0.4 mL of follicular fluid sample, 5 ng of each surrogate standard and 3 mL of ethyl acetate (containing 0.1% formic acid) were added. Then the material was extracted using a vortexer mixer for 5 min and centrifuged for 3 min at 4,000 rpm. After that, the supernatant was collected. This extraction procedure was carried out two times, and the extracts were pooled and concentrated until nearly dry under a gentle stream of nitrogen, then reconstituted in 50 µL of methanol and internal standards were added. Target chemicals in samples were analyzed using ultra-high performance liquid chromatography (Shimadzu) coupled with triple quadrupole mass spectrometry (Sciex 5500).

Chromatographic separation of target chemicals was applied by using an ACQUITY UPLC BEH Shield RP18 130A (100×2.1 mm, 1.7 µm; Waters). The mobile phase consisted of water (A) and methanol (B), flowing at a rate of 0.3 mL/min. The used gradients were as follows: *a*) 10% B held for 1 min and increased to 50% B in 2 min (linear), *b*) increased to 100% B in 5 min (linear) and held for 3 min, and *c*) changed to 10% B in 0.1 min and held for 3 min. The mass spectrometry was equipped with an atmospheric pressure chemical ionization probe and operated in multiple reaction monitoring modes. Table S1 presented the chemical-dependent mass spectral parameters and the limit of quantification (LOQ) for each chemical.

Quality control was conducted during the measurement procedure. First, to estimate potential background contamination, procedural blanks were analyzed in the analysis process (1 blank for each batch of 10 samples). In the final extracts of procedural blanks, BHA, BHT, BHT-OH, BHT-Q, BHT-quinol, and TBHQ were not measured, whereas BHT-CHO and BHT-COOH were measured at levels generally one order of magnitude lower than their levels as determined in follicular fluid. In calculating the final concentration, the blank concentration was subtracted from the measured concentrations for each target chemical in each sample. Moreover, to estimate the recovery of each target chemical from sample analysis, these chemicals with known amounts were spiked into quality control samples (mixture from all samples, $n=6$ replicates). The mean recoveries of these chemicals ranged from $70.0 \pm 8.3\%$ to $104.0 \pm 9.5\%$ after subtracting their original levels in the quality control samples. During the sample analysis procedure, the recoveries of the surrogate standard were $84 \pm 19\%$. The matrix effects for these chemicals ranged from $77.4 \pm 11.1\%$ to $110.9 \pm 6.8\%$. Detailed information about the supplier of the standard, recovery, matrix effect, and procedural blank for each target chemical is listed in Table S2.

Ovarian Reserve Markers

In our study, information about ovarian reserve makers was collected from the medical record. All these markers were measured usually 1 month (range: 0.5–5 months) before the oocyte retrieval when follicular fluid samples were collected. In the routine inspection, the AFC of each patient was determined at the early follicular phase (days 2–5) of a natural cycle by trained investigators using transvaginal ultrasound. AFC was the total number of antral follicles (2–9 mm in diameter) in each ovary during the early follicular phase as seen under transvaginal ultrasonography. Moreover, the hormone levels of each patient were measured at the endocrine laboratory of Peking University Third Hospital. Fasting blood samples from all subjects were collected during their early follicular phase of a natural menstrual cycle for basal hormone assay. Within 2 h after the sample collection, serum was separated (3,000 g, 10 min, 4°C) for subsequent analysis. The concentrations of FSH, luteinizing hormone (LH), and estradiol (E2) were measured by the chemiluminescence method (SIEMENS Immulite 2000),^{34,35} and AMH concentration was measured using the Ansh Lab assay kit.³⁶ The LOQs of FSH, LH, E2, and AMH were 0.1 mIU/mL, 0.1 mIU/mL, 73.4 pmol/L, and 0.1 ng/mL, respectively.

Covariates

Information about the basic and clinical characteristics of patients was collected from the medical record, including age, education level, weight (in kilograms), height (in meters), infertility type (primary infertility and secondary infertility), hormone levels [including AMH (in nanograms per milliliter), FSH (in milli-international units per milliliter), LH (in milli-international units per milliliter), and E2 (in picomoles per liter)], AFC, triglycerides (in millimoles per liter), and total cholesterol (in millimoles per liter). Body mass index (BMI) was calculated as the ratio of an individual's weight in kilograms divided by the height in meters squared. Education level included three levels: high school or below, university/college, and master's degree or above. Infertility type include primary infertility and secondary infertility. Primary infertility happens when a woman has never conceived earlier. Secondary infertility happens when a woman has conceived in the past but is currently unable to conceive again after 12 months or more of regular unprotected sexual intercourse.³⁷ Serum levels of triglycerides and total cholesterol were measured by using a clinical chemistry analyzer with enzymatic

assays of the glycerolphosphate oxidase–peroxidase aminophenazone method and the cholesterol oxidase–peroxidase aminophenazone method, respectively.^{38,39}

Statistical Analysis

We compared the women's age, BMI, education level, infertility type, and AFC, as well as the concentrations of AMH, FSH, LH, and E2 between the case and control groups. For variables with a normal distribution, mean with standard deviation (SD) were used as descriptive parameters, and *t*-tests were used to compare the difference between the two groups. For variables not following the normal distribution, median with interquartile ranges (IQR) and Mann–Whitney *U* tests were used for comparison. Measured frequencies for BHA, BHT-CHO, BHT-COOH, BHT-OH, and BHT-Q in the follicular fluid between the two groups were compared using χ^2 tests. The exposure level of each chemical in the case and control groups was described with median with IQR and compared with the Mann–Whitney *U*-test. Concentrations below the LOQ were assigned a value equal to one-half the LOQ.

The concentration of each target chemical was categorized into three levels (low, middle, and high exposure levels) according to the tertiles calculated based on all participants. The detailed concentration ranges of the levels for each chemical are shown in Table S3. Unconditional logistic regression models were applied to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) of DOR in the middle and high exposure levels compared with the low exposure level. Moreover, adjusted ORs and 95% CIs were calculated after adjusting for potential confounders, including age (23–29, 30–34, and 35–44 y), BMI (<18.5, 18.5–24, and >24 kg/m²), education (high school or below, university/college, and master's degree or above), and infertility type (primary infertility and secondary infertility), as well as triglycerides (≤ 0.67 , 0.68–0.98, 0.99–1.18, and ≥ 1.19 mmol/L) and total cholesterol (≤ 3.80 , 3.81–4.28, 4.29–4.84, and ≥ 4.85 mmol/L with the levels divided according to the quartiles). All these potential confounders were adjusted as categorical variables in multivariate logistic regression models. The statistical significance of the linear trend for the ORs was tested with a separate model where the categorical variables were treated as ordinal variables in the regression models. We also conducted the related analyses mentioned above for the sum of five BHT metabolites of BHT-OH, BHT-CHO, BHT-COOH, BHT-Q, and BHT-quinol ($\Sigma 5$ metabolites).

Moreover, sensitivity analysis was conducted by using the exposure levels categorized by another method, of which the exposure levels were categorized according to the tertiles calculated only including controls for analysis. The detailed concentration ranges of the exposure levels used in sensitivity analysis are shown in Table S3. Given that age is an independent risk factor for DOR and that the risk of DOR remarkably increased for women ≥ 35 years of age,^{3,9} we also conducted a subgroup analysis according to the women's age (<35 y and ≥ 35 y). In multivariate logistic regression models of subgroup analysis, potential confounders of BMI, education, infertility type, triglycerides, and total cholesterol were adjusted as categorical variables in each of the strata. In addition, In the strata of <35 years of age, age was also adjusted as a categorical variable with two levels of 23–29 and 30–34 y, whereas in the strata of ≥ 35 years of age, age was not further adjusted (only 7 women were 41–44 y old). Finally, Spearman's coefficients were used to assess the correlations between SPAs and their metabolites with markers of ovarian reserve.

Given that the measured frequency of some target chemicals was very low (e.g., <10%), we described only their measured frequency and did not conduct other analyses for them. R statistical software (version 4.1.1; R Development Core Team) was used

Table 1. Characteristics of infertile women with and without DOR: a case–control study conducted in China during 2019–2020.

	Overall (<i>N</i> = 181) [mean ± SD, <i>n</i> (%), or median (IQR)] ^a	DOR cases (<i>N</i> = 63) [mean ± SD, <i>n</i> (%), or median (IQR)]	Controls (<i>N</i> = 118) [mean ± SD, <i>n</i> (%), or median (IQR)]	<i>p</i> -Value
Age (y)				
Mean ± SD	32.6 ± 4.4	35.1 ± 4.4	31.1 ± 3.7	<0.001
23–29	49 (27.1)	6 (9.5)	43 (36.5)	<0.001
30–34	72 (39.8)	21 (33.3)	51 (43.2)	—
35–44	60 (33.1)	36 (57.2)	24 (20.3)	—
BMI (kg/m ²)				
Mean ± SD	21.6 ± 2.5	21.8 ± 2.6	21.5 ± 2.4	0.419
<18.5	17 (9.6)	5 (8.1)	12 (10.3)	0.863
18.5–24	111 (62.4)	39 (62.9)	72 (62.1)	—
>24	50 (28.0)	18 (29.0)	32 (27.6)	—
Education				
High school or below	41 (23.6)	14 (22.6)	27 (24.1)	0.506
University/college	100 (56.2)	34 (54.8)	66 (58.9)	—
Master's degree or above	33 (19.0)	14 (22.6)	19 (17.0)	—
Infertility type				
Primary infertility	126 (69.6)	37 (58.7)	89 (75.4)	0.023
Secondary infertility	55 (30.4)	26 (41.3)	29 (24.6)	—
Triglycerides (mmol/L)				
Median (IQR)	1.0 (0.7–1.2)	1.0 (0.7–1.2)	0.9 (0.7–1.2)	0.852
≤0.67	47 (26.0)	14 (22.2)	33 (28.0)	0.315
0.68–0.98	48 (26.5)	15 (23.8)	33 (28.0)	—
0.99–1.18	41 (22.7)	20 (31.7)	21 (17.8)	—
≥1.19	45 (24.9)	14 (22.2)	31 (26.3)	—
Total cholesterol (mmol/L)				
Median (IQR)	4.3 (3.8–4.8)	4.2 (3.7–4.8)	4.3 (3.9–4.9)	0.246
≤3.80	46 (25.4)	19 (30.2)	27 (22.9)	0.230
3.81–4.28	48 (26.5)	18 (28.6)	30 (25.4)	—
4.29–4.84	43 (23.8)	12 (19.0)	31 (26.3)	—
≥4.85	44 (24.3)	14 (22.2)	30 (25.4)	—
AFC (number) ^b	8.0 (5.5–12.1)	4.5 (3.0–6.0)	11.0 (8.0–14.0)	<0.001
AMH (ng/mL) ^b	1.3 (0.6–2.8)	0.5 (0.27–0.7)	2.0 (1.3–3.5)	<0.001
FSH (mIU/mL) ^b	7.5 (6.1–10.0)	11.4 (8.1–13.2)	6.9 (5.7–8.0)	<0.001
LH (mIU/mL) ^b	3.9 (2.5–5.4)	4.1 (2.9–5.4)	3.7 (2.4–5.2)	0.196
E2 (pmol/L) ^b	154.0 (121.5–192.0)	154.0 (114.8–204.5)	155.4 (126.0–188.5)	0.821

Note: —, not applicable; AFC, antral follicle count; AMH, anti-Müllerian hormone; BMI, body mass index; DOR, diminished or decreased ovarian reserve; E2, estradiol; FSH, follicle-stimulating hormone; IQR, interquartile range; LH, luteinizing hormone; SD, standard deviation.

^aData for some characteristic variables was missing: 3 for BMI (1 in case group and 2 in control group), 7 for education (1 in case group and 6 in control group), 6 for AMH (2 in case group and 4 in control group), 1 for FSH (1 in case group), and 3 for E2 (3 in case group).

^bMedian (interquartile range) was shown for these characteristics.

for analysis. A two-tailed $p < 0.05$ was considered statistically significant. Missing data were omitted from the related analyses.

Results

Study Population Characteristics

A total of 181 women were included in the final analysis, including 63 women with DOR (case group) and 118 women without DOR (control group). Characteristics and biomarkers of ovarian reserve of the two groups are summarized in Table 1. The BMI, education degree, triglycerides level, and total cholesterol level were similar between the two groups. Compared with controls, women with DOR were likely to be older, experienced secondary infertility, had lower levels of AFC and AMH, and had higher levels of FSH.

Measured Frequencies of SPAs and the Major Metabolites

The measured frequencies of SPAs and their major metabolites in follicular fluid samples are presented in Table 2. Among the three target SPAs, BHT is the most abundant in follicular fluid, with a measured frequency of 40.3%, but the measured frequencies for BHA and TBHQ were very low. For the five target metabolites of BHT, BHT-COOH was the most dominant compound, with a measured frequency of 100%, followed by BHT-Q, BHT-CHO, BHT-OH, and BHT-quinol. Compared with the control group, the measured frequencies of BHT-OH (57.1% vs. 40.7%, $p = 0.050$)

and BHT-Q (79.4% vs. 56.8%, $p = 0.004$) were significantly higher than those in the case group.

Concentrations of BHT and Its Metabolites

Table 3 presents the follicular fluid concentrations of BHT and its metabolites in the case and control groups. No significant

Table 2. Measured frequencies of SPAs and their metabolites (ng/mL) in follicular fluid for women with and without DOR.

	Overall (<i>N</i> = 181) [<i>n</i> (%)]	DOR cases (<i>N</i> = 63) [<i>n</i> (%)]	Controls (<i>N</i> = 118) [<i>n</i> (%)]	<i>p</i> -Value
SPAs				
BHT	73 (40.3)	26 (41.3)	47 (39.8)	0.977
BHA	3 (1.7)	0 (0.0)	3 (2.5)	0.506
TBHQ	4 (2.2)	1 (1.6)	3 (2.5)	0.999
BHT metabolites				
BHT-OH	84 (46.4)	36 (57.1)	48 (40.7)	0.050
BHT-CHO	104 (57.5)	40 (63.5)	64 (54.2)	0.297
BHT-COOH	181 (100.0)	63 (100)	118 (100.0)	0.999
BHT-Q	117 (64.6)	50 (79.4)	67 (56.8)	0.004
BHT-quinol	89 (49.2)	37 (58.7)	52 (44.1)	0.085

Note: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BHT-CHO, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; BHT-COOH, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid; BHT-OH, 3,5-di-*tert*-butyl-4-hydroxy-benzylalcohol; BHT-Q, 2,6-di-*tert*-butyl-1,4-benzoquinone; BHT-quinol, 2,6-di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one; DOR, diminished or decreased ovarian reserve; SPA, synthetic phenolic antioxidant; TBHQ, *tert*-butylhydroquinone.

Table 3. Follicular fluid concentrations of BHT and its metabolites (ng/mL) for women with and without DOR.

	Overall (<i>N</i> = 181) [median (IQR)]	DOR cases (<i>N</i> = 63) [median (IQR)]	Controls (<i>N</i> = 118) [median (IQR)]	<i>p</i> -Value
BHT	0.09 (0.09–0.48)	0.09 (0.09–0.72)	0.09 (0.09–0.45)	0.424
BHT metabolites				
BHT-OH	0.01 (0.01–0.05)	0.03 (0.01–0.05)	0.01 (0.01–0.05)	0.161
BHT-CHO	0.26 (0.03–0.90)	0.49 (0.03–1.50)	0.12 (0.03–0.68)	0.041
BHT-COOH	0.30 (0.21–0.47)	0.45 (0.24–0.63)	0.28 (0.20–0.36)	<0.001
BHT-Q	0.25 (0.01–0.89)	0.70 (0.10–1.30)	0.13 (0.01–0.70)	<0.001
BHT-quinol	0.02 (0.02–0.64)	0.25 (0.02–0.67)	0.02 (0.02–0.60)	0.072
Σ5metabolites	1.35 (0.63–2.69)	1.79 (1.17–4.04)	1.01 (0.41–2.33)	<0.001

Note: BHT, butylated hydroxytoluene; BHT-CHO, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; BHT-COOH, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid; BHT-OH, 3,5-di-*tert*-butyl-4-hydroxy-benzylalcohol; BHT-Q, 2,6-di-*tert*-butyl-1,4-benzoquinone; BHT-quinol, 2,6-di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one; DOR, diminished or decreased ovarian reserve; IQR, interquartile range; Σ5metabolites, sum of five BHT metabolites, including BHT-OH, BHT-CHO, BHT-COOH, BHT-Q, and BHT-quinol.

difference was found between the two groups for the concentration of BHT. The concentrations of BHT-CHO, BHT-COOH, and BHT-Q in the case group were significantly higher than those in the control group. Moreover, the concentrations of these three metabolites were higher than that of BHT (the parent material). When comparing the sum concentration of the five BHT metabolites, we found the concentration of Σ5 metabolites in the case

group was significantly higher than that in the control group (1.79 ng/mL vs. 1.01 ng/mL, *p* < 0.001).

BHT and Its Metabolites with DOR

The adjusted ORs for DOR were significantly increased for the high exposure levels of BHT-CHO [OR = 3.19 (95% CI: 1.22, 8.31),

Table 4. Unconditional logistic regression models for the association of BHT and its metabolites with DOR: a case–control study conducted in China during 2019–2020.

	Overall (<i>N</i> = 181) [<i>n</i> (%)]	DOR cases (<i>N</i> = 63) [<i>n</i> (%)]	Controls (<i>N</i> = 118) [<i>n</i> (%)]	Crude OR ^a	Crude <i>p</i> -Value ^a	Adjusted OR ^b	Adjusted <i>p</i> -Value ^b
BHT							
Low	108 (59.7)	37 (58.7)	71 (60.2)	1 (Ref)		1 (Ref)	
Middle	13 (7.2)	1 (1.6)	12 (10.2)	0.16 (0.02, 1.28)	0.084	0.27 (0.03, 2.48)	0.246
High	60 (33.1)	25 (39.7)	35 (29.7)	1.37 (0.72, 2.62)	0.341	1.11 (0.49, 2.51)	0.807
<i>p</i> _{Trend}					0.428		0.865
BHT-OH							
Low	97 (53.6)	27 (42.9)	70 (59.3)	1 (Ref)		1 (Ref)	
Middle	24 (13.3)	11 (17.5)	13 (11.0)	2.19 (0.88, 5.49)	0.093	1.20 (0.38, 3.75)	0.755
High	60 (33.1)	25 (39.7)	35 (29.7)	1.85 (0.94, 3.65)	0.075	1.71 (0.72, 4.07)	0.227
<i>p</i> _{Trend}					0.063		0.230
BHT-CHO							
Low	77 (42.5)	23 (36.5)	54 (45.8)	1 (Ref)		1 (Ref)	
Middle	44 (24.3)	13 (20.6)	31 (26.3)	0.98 (0.44, 2.22)	0.970	1.31 (0.45, 3.77)	0.618
High	60 (33.1)	27 (42.9)	33 (28.0)	1.92 (0.95, 3.89)	0.069	3.19 (1.22, 8.31)	0.018
<i>p</i> _{Trend}					0.075		0.017
BHT-COOH							
Low	60 (33.1)	17 (27.0)	43 (36.4)	1 (Ref)		1 (Ref)	
Middle	61 (33.7)	10 (15.9)	51 (43.2)	0.50 (0.21, 1.20)	0.118	0.55 (0.19, 1.63)	0.282
High	60 (33.1)	36 (57.1)	24 (20.3)	3.79 (1.77, 8.14)	0.001	4.73 (1.63, 13.71)	0.004
<i>p</i> _{Trend}					<0.001		0.004
BHT-Q							
Low	64 (35.4)	13 (20.6)	51 (43.2)	1 (Ref)		1 (Ref)	
Middle	57 (31.5)	19 (30.2)	38 (32.2)	1.96 (0.86, 4.46)	0.108	1.56 (0.58, 4.19)	0.382
High	60 (33.1)	31 (49.2)	29 (24.6)	4.19 (1.90, 9.26)	<0.001	4.48 (1.69, 11.86)	0.003
<i>p</i> _{Trend}					<0.001		0.002
BHT-quinol							
Low	92 (50.8)	26 (41.3)	66 (55.9)	1 (Ref)		1 (Ref)	
Middle	29 (16.0)	11 (17.5)	18 (15.3)	1.55 (0.65, 3.73)	0.326	1.46 (0.49, 4.37)	0.499
High	60 (33.1)	26 (41.3)	34 (28.8)	1.94 (0.98, 3.84)	0.057	1.76 (0.76, 4.11)	0.188
<i>p</i> _{Trend}					0.055		0.179
Σ5metabolites							
Low	60 (33.1)	9 (14.3)	51 (43.2)	1 (Ref)		1 (Ref)	
Middle	61 (33.7)	27 (42.9)	34 (28.8)	4.50 (1.88, 10.74)	0.001	4.11 (1.44, 11.75)	0.008
High	60 (33.1)	27 (42.9)	33 (28.0)	4.64 (1.94, 11.09)	0.001	5.51 (1.81, 16.77)	0.003
<i>p</i> _{Trend}					0.001		0.003

Note: The concentrations of BHT and its metabolites were categorized into three levels (low, middle, and high) according to their tertiles calculated based on all participants. Detailed information about the tertile definitions are presented in Table 3. BHT, butylated hydroxytoluene; BHT-CHO, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; BHT-COOH, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid; BHT-OH, 3,5-di-*tert*-butyl-4-hydroxy-benzylalcohol; BHT-Q, 2,6-di-*tert*-butyl-1,4-benzoquinone; BHT-quinol, 2,6-di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one; BMI, body mass index; DOR, diminished or decreased ovarian reserve; OR, odds ratio; Ref, reference; Σ5metabolites, sum of five BHT metabolites, including BHT-OH, BHT-CHO, BHT-COOH, BHT-Q, and BHT-quinol.

^aUnconditional logistic regression model without adjustments.

^bUnconditional logistic regression model with adjustments of age, BMI, education, infertility type, triglycerides, and total cholesterol.

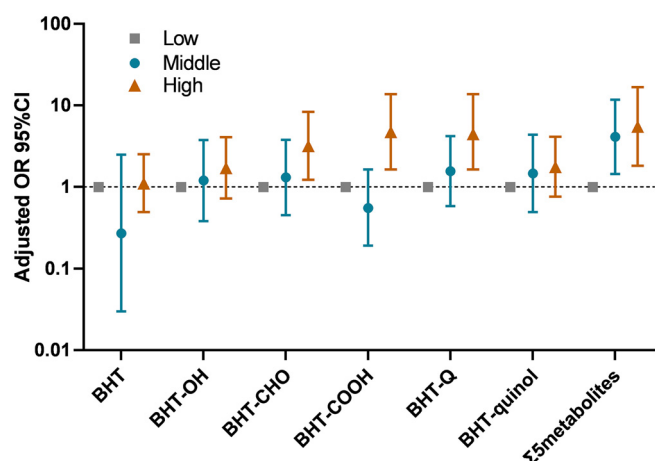


Figure 1. The relationships of BHT and its metabolites with DOR (the corresponding numeric results are presented in Table 4). Adjusted ORs and 95% CIs were calculated using unconditional logistic regression models after adjusting for age, BMI, education, infertility type, triglycerides, and total cholesterol. Detailed information about the tertile definitions was presented in Table S3. Note: BHT, butylated hydroxytoluene; BHT-CHO, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; BHT-COOH, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid; BHT-OH, 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol; BHT-Q, 2,6-di-*tert*-butyl-1,4-benzoquinone; BHT-quinol, 2,6-di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one; BMI, body mass index; CI, confidence interval; DOR, diminished or decreased ovarian reserve; OR, odds ratio; Σ5metabolites, sum of five BHT metabolites, including BHT-OH, BHT-CHO, BHT-COOH, BHT-Q, and BHT-quinol.

$p = 0.018$; Table 4 and Figure 1], BHT-COOH [OR = 4.73 (95% CI: 1.63, 13.71), $p = 0.004$], and BHT-Q [OR = 4.48 (95% CI: 1.69, 11.86), $p = 0.003$], compared with their low exposure levels. Moreover, compared with the low level of Σ5metabolites, adjusted ORs for DOR were increased both at the middle [4.11 (95% CI: 1.44, 11.75), $p = 0.008$] and high levels [5.51 (95% CI: 1.81, 16.77), $p = 0.003$], showing a statistically significant concentration–

Table 5. Spearman correlation between ovarian reserve markers and BHT, as well as its metabolites.

	AFC	AMH	FSH	LH	E2
BHT					
<i>r</i>	−0.113	−0.077	0.233	0.023	−0.210
<i>p</i> -Value	0.133	0.312	0.002	0.762	0.005
BHT-OH					
<i>r</i>	−0.094	−0.041	0.206	0.048	−0.168
<i>p</i> -Value	0.211	0.587	0.006	0.527	0.025
BHT-CHO					
<i>r</i>	−0.181	−0.214	0.104	−0.076	−0.115
<i>p</i> -Value	0.016	0.004	0.164	0.316	0.125
BHT-COOH					
<i>r</i>	−0.319	−0.371	0.321	−0.095	−0.144
<i>p</i> -Value	<0.001	<0.001	<0.001	0.208	0.056
BHT-Q					
<i>r</i>	−0.279	−0.364	0.258	−0.074	−0.081
<i>p</i> -Value	<0.001	<0.001	<0.001	0.329	0.285
BHT-quinol					
<i>r</i>	−0.134	−0.239	0.161	−0.105	−0.062
<i>p</i> -Value	0.074	0.001	0.031	0.165	0.408
Σ5metabolites					
<i>r</i>	−0.334	−0.406	0.244	−0.090	−0.120
<i>p</i> -Value	<0.001	<0.001	0.001	0.232	0.112

Note: AFC, antral follicle count; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; BHT, butylated hydroxytoluene; BHT-CHO, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; BHT-COOH, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid; BHT-OH, 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol; BHT-Q, 2,6-di-*tert*-butyl-1,4-benzoquinone; BHT-quinol, 2,6-di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one; E2, estradiol; LH, luteinizing hormone; Σ5metabolites, sum of five BHT metabolites, including BHT-OH, BHT-CHO, BHT-COOH, BHT-Q, and BHT-quinol.

response relationship ($p_{\text{Trend}} = 0.003$). We also conducted a sensitivity analysis according to the exposure levels categorized by tertiles of BHT and its metabolites calculated based on their concentrations of controls. The cutoff values of these tertiles were a little lower than those calculated based on all participants, especially the high levels (Table S3). The main results of the sensitivity analysis were comparable to the results conducted from the tertiles calculated based on all participants (Table S4). Moreover, we conducted the subgroup analysis according to age. We found that BHT-OH, BHT-CHO, BHT-COOH, and BHT-Q, as well as Σ5metabolites, were associated with DOR among women <35 y old, and BHT-CHO was associated with DOR among women ≥35 y old (Table S5).

Ovarian Reserve Markers with BHT and Its Metabolites

We found ovarian reserve markers were statistically significantly associated with BHT and its metabolites (Table 5). AFC and AMH were negatively associated with levels of BHT-CHO, BHT-COOH, BHT-Q, BHT-quinol, and Σ5metabolites. FSH was positively associated with BHT, BHT-OH, BHT-COOH, BHT-Q, BHT-quinol, and Σ5metabolites. E2 was negatively associated with BHT and BHT-OH.

Discussion

Main Findings

To the best of our knowledge, this is the first study to explore the relationship between SPAs exposure levels and DOR. We found that concentrations of BHT-CHO, BHT-COOH, BHT-Q, and Σ5metabolites were significantly higher for women with DOR compared with the controls. The concentrations of these three chemicals are higher than that of BHT, which is their parent material. We found BHT metabolites were associated with DOR. Consistent with these results, we also found these BHT metabolites are negatively correlated with AFC, as well as AMH, and positively correlated with FSH. Moreover, our study reports the exposure levels of BHT, BHA, TBHQ, and their metabolites in follicle fluid for reproductive-aged women. Among the three target SPAs, BHT is the most abundant in follicular fluid, whereas BHA and TBHQ were lowly detected. Among the five BHT metabolites, BHT-COOH is the most detected compound, with a measured frequency of 100%, followed by BHT-Q, BHT-CHO, BHT-OH, and BHT-quinol.

Associations between BHT Metabolites and DOR

As far as we know, no previous study has estimated the potential adverse effects of SPAs and their metabolites on the ovarian reserve or female reproductive health. This case–control study indicates higher levels of the BHT metabolites, especially BHT-CHO, BHT-COOH, and BHT-Q, are associated with DOR in reproductive-aged women. Consistent with these findings, we also found these BHT metabolites were correlated with higher levels of FSH, as well as lower levels of AMH and AFC, which are the indications of DOR. Unlike the BHT metabolites, we did not find an association between BHT itself and DOR, which indicated that, compared with BHT, its metabolites may have more adverse effects on ovarian reserve. This is consistent with the findings of an animal study that the toxic effects of BHT are thought to be caused by its metabolites rather than by the parent compound.⁴⁰ Moreover, results of a subgroup analysis suggested that different BHT metabolites may have different effects on women <35 years of age and ≥35 years of age.

Whether BHT metabolites are involved in the development of DOR is still unknown. We found BHT and its metabolites were associated with concentrations of FSH and AMH in serum, suggesting that they are endocrine disruptors. This is consistent with the findings of a previous study that BHT exposure could repress

the expression of endogenous estrogen-responsive genes, the progesterone receptor (PGR), growth regulation by estrogen in breast cancer 1 (GREB1), and natriuretic peptide C (NPPC) in a human uterine cell line.²⁸ An animal study found that BHT exposure during early pregnancy could decrease the expression of endometrial decidual markers, up-regulate the levels of serum E2 and progesterone as well as the expression levels of uterus estrogen receptor α and PGR, and inhibit decidualization of the mouse endometrium.⁴¹ A number of epidemiological and experimental studies indicate that exposure to endocrine disruptors (such as chemicals with anti-androgenic or estrogenic activity) could accelerate follicle exhaustion, contribute to ovarian aging, and increase the risk of DOR.^{42–44} Therefore, BHT metabolites may potentially diminish the ovarian reserve by interfering with the homeostasis of the endocrine system. In addition, *in vitro* studies showed that BHT metabolites may cause oxidative stress in the Burkitt's lymphoma cell line BJAB and the human myelogenous leukemia cell line HL 60 cell by influencing the mitochondrial electron transport chain.^{30,31} Oxidative stress resulting from BHT metabolites may further accelerate the decline in ovarian follicles by increasing the recruitment of follicles into the growing pool and causing apoptosis at later stages of follicular development.⁴⁵ Moreover, BHT metabolites could cause DNA damage directly or via induction of oxidative stress.^{30,31} Increased DNA damage may lead to genomic abnormalities and eventually lead to cellular apoptosis by various pathways, including activation of p53/p63, ceramide, caspase 2, and Bax.^{46,47} The DNA damage and apoptosis of granulosa cells and oocytes contribute to the pathogenesis of ovarian aging and DOR.⁴⁸ Therefore, exposure to increased levels of BHT metabolites may lead to DOR by causing oxidative stress, DNA damage, and cell apoptosis of granulosa cells and oocytes. However, further studies are needed to elucidate the underlying mechanisms of how BHT affects the development of DOR.

SPAs Exposure Levels in Follicular Fluid

In our study, SPAs and their metabolites were measured in human follicular fluid, suggesting these chemicals could pass through the blood–follicle barrier, enter the intra-ovarian environment, and directly affect the oocytes. BHT is one of the most widely used SPAs.¹³ In the natural environment and in organisms, BHT is mainly transformed to BHT-OH, BHT-CHO, and BHT-COOH by continuous oxidation of the para-methyl group (pathway 1) and to BHT-Q, as well as BHT-quinol, by oxidation of the aromatic ring system (pathway 2).^{13,14,49} In our study, the metabolite BHT-COOH was measured in all follicular fluid samples, indicating that BHT-COOH may be the dominant metabolite of BHT in humans and could be recognized as a potential biomarker of exposure to BHT. This is consistent with the findings from human urine samples^{19,23} and fingernails²⁵ of different populations in different countries. These findings also indicate that the dominant metabolic pathway of BHT in humans may be the oxidation of the para-methyl group by yielding BHT-COOH.²³

Moreover, we found the measured concentrations and frequencies of BHT-COOH were higher than those of BHT. A previous study indicated that the area under the concentration–time curve from 0 to 120 h (AUC_{0-120h}) value of BHT-COOH in blood was $9.86 \text{ h} \times \mu\text{g/mL}$, which was ~ 30 times higher than that of BHT ($0.30 \text{ h} \times \mu\text{g/mL}$). These results suggest that BHT in the blood may be excreted in a short time, whereas BHT-COOH preferentially accumulates in the blood. To some extent, the excretion patterns of BHT and BHT-COOH in the follicular fluid may be similar to those in the blood.

The median concentration of BHT-COOH in the follicle fluid (0.30 ng/mL) is similar to that measured in urine from the Chinese population during 2010–2012 (0.26 ng/mL)¹⁹ and lower than that

in urine from the U.S. population in 2018 (0.66 ng/mL)²³ and the Japanese population during 2010–2012 (3.86 ng/mL).¹⁹ The measured frequencies and concentrations of TBHQ, BHA, BHT, BHT-CHO, BHT-OH, BHT-Q, and BHT-quinol in follicular fluid were lower in this study than the measured levels in other samples reported in previous studies. For example, BHT-CHO was measured in 57.5% of follicular fluid but in 100% of maternal plasma,²² 92% of placenta,²² 90% of breast milk,²⁴ and 59.9%–98% of urine samples.^{19,23} The follicle fluid concentration of BHT-CHO (0.26 ng/mL) is also lower than that measured in maternal breast milk (0.51 ng/mL)²⁴ and urine (0.61 ng/mL)¹⁹ for the Chinese population. These discrepancies may be due to the difference in samples and populations. The blood–follicle transfer efficiencies of these chemicals, especially TBHQ and BHA, may be relatively low.

Compared with populations in other studies, the population in our study is at a relatively low exposure level of BHT. However, robust associations were found between BHT metabolites and DOR among our study population, suggesting that BHT metabolites may have adverse effects on the ovarian reserve even at low concentrations. Therefore, it should be noted that most reproductive-aged women may be exposed to a BHT level that potentially diminishes their ovarian reserve and reduces their fecundity.

Strengths and Limitations

Our study has several strengths. First, to the best of our knowledge, this is the first study that explored the presence of BHT, BHA, TBHQ, and their major metabolites in human follicular fluid. Follicular fluid is the microenvironment that growing oocytes and their surrounding somatic cells directly contact and which is critical for oocyte development.⁵⁰ The follicular fluid concentrations of SPAs and metabolites, compared with other samples (e.g., serum, urine, fingernails), could accurately reflect the actual exposure level of the oocytes and the biologically effective/target organ doses. Second, we provide the epidemiologic evidence that BHT metabolites, especially BHT-CHO, BHT-COOH, and BHT-Q, may play a role in the quality and quantity of the ovarian reserve of reproductive-aged women. These findings not only provide possible evidence for the reproductive toxicity of BHT but also give new clues to explore the related mechanism and pathway for DOR.

Our study also has some limitations. First, we cannot conclude the causal relationship between SPAs exposure and DOR given that a case–control design is used in our study. Further prospective epidemiological studies with larger samples and well-designed experimental studies are still warranted to verify our findings. Second, residual confounding cannot be ruled out given that we did not have data on covariates, including physical activities, diet, and nutrition supplements. Moreover, residual confounding by age still exists given that we adjusted age as a categorical variable in the models. Third, the exact mechanisms for the relationship are still unclear, although our results suggest SPAs and their metabolites are a group of endocrine-disrupting chemicals that may potentially impact the fecundity of reproductive-aged women. *In vitro* and *in vivo* experiments of exposure to these metabolites are warranted to explore the detailed mechanisms behind the associations. Fourth, the chemiluminescence method used to measure the hormone concentrations was not validated against the liquid chromatographic–mass spectrometric analysis method. Finally, the combined effects and related mechanisms of other endocrine-disrupting chemicals (e.g., perfluoroalkyl sulfonate) with BHT metabolites on DOR were not estimated in our study.

Conclusion

In the present study, we examined the association between SPAs and their metabolites with DOR. We found that high levels of

BHT metabolites, especially BHT-CHO, BHT-COOH, and BHT-Q, were associated with DOR, as well as with the related indications of DOR. Moreover, our study showed SPAs and their metabolites could be measured in follicular fluid, indicating that these chemicals can cross the human ovarian blood–follicle barrier, enter follicular fluid, and may have direct effects on oocytes. Increasing concerns should be received about the potential adverse effects of BHT on ovarian reserve and the fertility of women.

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